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Hyperthermophilic composting accelerates the removal of antibiotic resistance genes and mobile genetic elements in sewage sludge

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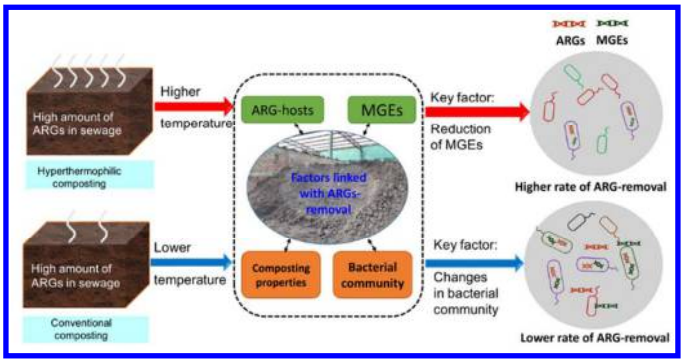
Abstract

Composting is an efficient way to convert organic wastes into fertilizers. However, waste materials often contain high amount of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) that can reduce the efficacy of antibiotic treatments when transmitted to humans. Because conventional composting often fails to remove these compounds, we evaluated if hyperthermophilic composting with elevated temperature is more efficient at removing ARGs and MGEs, and explored the underlying mechanisms of ARG-removal between two composting methods. We found that hyperthermophilic composting removed ARGs and MGEs more efficiently than conventional composting (89% and 49%, respectively). Furthermore, half-lives of ARGs and MGEs were lower in hyperthermophilic compared to conventional composting (67% and 58%, respectively). More efficient removal of ARGs and MGEs was associated with higher reduction in bacterial abundances and diversity of potential ARG hosts. Partial least squares path modeling suggested that reduction of MGEs played a key role in ARG-removal in hyperthermophilic composting, while ARG reduction was mainly driven by changes in bacterial community composition under conventional composting. Together these results suggest that hyperthermophilic composting can significantly enhance the removal of ARGs and MGEs and that the mechanisms of ARG and MGE removal can depend on composting temperature.

Keywords: Composting, biosolids, temperature, bacterial communities, ARGs

TOC art (approx. 8.47 cm by 4.76 cm)



Introduction

There is an urgent need to reduce the overuse of chemical fertilizers for economic and environmental reasons[1, 2]. The use of manure-based organic fertilizers are a promising alternative to chemical fertilizers and at the same time provide an efficient mean to process organic wastes. However, therein lies a potential risk: waste products often contain high amount of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs)[3] that can reduce the efficacy of antibiotic therapies when transmitted to humans[4]. This is because ARGs often make pathogenic bacteria resistant to clinically used antibiotics[5] and mobile genetic elements (MGEs), such as plasmids and transposons, can mobilize ARGs between different environment via horizontal gene transfer between different bacteria[6]. Current research suggests that antibiotic resistance genes have become more common in the environment due to heavy use of antibiotics in livestock industries[3] and enrichment of ARGs in aquatic environments[7-9]. For example, wastewater treatment plants (WWTPs), and specifically activated sludge, are important reservoirs for ARGs[10, 11] where high bacterial abundances and diversity is expected to further promote the horizontal gene transfer of ARGs[11]. Direct land application of sludge waste as soil amendment (organic fertilizer) is likely to increase the probability of introducing ARGs into soil bacterial communities[12, 13] from which they could be transferred to vegetables and humans[14]. Introducing ARGs to soil could also elevate the risk of transferring ARGs between non-pathogenic and human pathogenic bacteria via horizontal gene transfer[15, 16]. As a result, correct treatment of sewage sludge is very important to reduce the potential risks of spreading ARGs across agricultural environments.

Various solid waste management practices have been developed for reducing the abundance of ARGs[17, 18]. For example, bio-drying aeration strategies have been shown to significantly decrease the tetracycline resistance and class 1 integron integrase (*intI1*) genes in the sludge[19]. Similarly, the addition of zero-valent iron to anaerobic co-digestion of sludge and kitchen waste has also been demonstrated to lead to reduction in the amount of ARGs[20]. Moreover, high temperatures (55 °C vs. 35 °C) has been shown to be important in reducing ARGs more efficiently from anaerobic

digestion sludge[17]. Yet, increasing evidence suggests that conventional aerobic composting and anaerobic digestion do not effectively control the proliferation and diffusion of ARGs and MGEs[21-24]. Furthermore, reduction of ARGs is often observed only on the short-term and ARGs typically rebound after completion of the treatment[19, 21]. One potential explanation for this is that ARGs can be located on mobile genetic elements, which can promote their transfer between different bacterial strains and potential ARGs hosts[25]. Another possible explanation is that thermophilic composting temperature (approximately 55-70 °C) is not high enough for the degradation of the DNA that contain ARGs and/or MGEs even though some of the potential hosts are killed[21]. These few examples suggest that composting is a complex process and that we are still lacking a mechanistic understanding of ARG-removal[21, 26]. For example, it is not clear if the ARG-removal is driven by (1) changes in abundances or community composition of bacteria, (2) physicochemical properties of the compost or (3) both of them[23, 26, 27]. As a result, a better understanding of the elevated temperature for bacterial communities and gene abundances during the composting is vital for developing more efficient techniques for the removal of ARGs and MGEs[21, 25].

Here we evaluated the performance of hyperthermophilic composting for the removal of ARGs and MGEs from activated sewage sludge. The hyperthermophilic aerobic composting technique was first developed by Oshima[28]. During the fermentation process, composting temperatures reach extremely high temperatures of up to 90 °C without exogenous heating, which is 20-30 °C higher compared to conventional composting[28]. Hyperthermophilic composting has also some other prominent features, such as high bioconversion efficiency[29], and has been shown to be associated with distinct microbial communities[30]. However, there are no published studies on the impact of hyperthermophilic composting on ARGs abundances, and as a result, it is unclear if hyperthermophilic composting is efficient at removing both ARGs and MGEs compared to conventional composting. Here we studied this experimentally by directly comparing these two composting methods. Furthermore, we tried to achieve a more mechanistic understanding of how ARGs are sustained in

the environment by temporally sampling their potential bacterial hosts and looking changes in the entire bacterial community by applying quantitative PCR (qPCR) and Illumina sequencing of bacterial 16S rRNA genes. We hypothesized that: (i) hyperthermophilic composting is more efficient at removing both ARGs and MGEs than conventional composting; (ii) the two composting methodologies will select distinct bacterial communities during the composting; (iii) higher efficiency of ARG-removal is associated with a reduced frequency of potential ARG hosts; and/or, (iv) limits the changes of horizontal gene transfer by more efficiently removing MGEs.

Materials and methods

Conventional and hyperthermophilic aerobic composting setup

Here we compared how two composting processes, conventional and hyperthermophilic aerobic composting, affect the abundance of ARGs, MGEs and the diversity and composition of bacterial communities. Our experiments were carried out in a full-scale sludge hyperthermophilic aerobic composting plant located in Shunyi district, Beijing, China. The detailed process of hyperthermophilic aerobic composting technology has been described previously by Liao *et al.*[31]. Briefly, raw materials including dewatered sewage sludge (with around 75% moisture content; Shunyi WWTPs, Beijing, China) and composting end-products (with around 40% moisture content including 5% rice husk) from the previous composting round were first thoroughly mixed with a ratio of 1:3 (v/v) to adjust the initial moisture content to approximately 60% (with C:N ratio around 8). The compost mixture (approximately 200 tons) was then loaded to the fermentation compartment (8.5 m length, 6 m width and 3.2 m height) up to 2.5 m in height. Forced aeration via two PVC tubes running underground from bottom to the top of the composting pile were supplied according to aeration needs of hyperthermophilic composting[31]. To mix the compost substrate well and to reduce pile-edge effects, mechanical turning of composting material was performed at every four days using pile-specific forklifts to prevent cross-contamination between the piles. Conventional composting followed a

previously described protocol by Tortosa *et al.*[32]. Briefly, the same raw materials were used for conventional and hyperthermophilic composting to build a trapezoidal pile of about 20 tons. Fresh air was supplied naturally without forced aeration by turning the composting material at every two days during the composting process. Hyperthermophilic composting takes normally 25 days according to the experience of the compost factory (Liao; personal communication). In contrast, conventional composting takes around 45 days. Hence, both composting treatments were run synchronously for 45 days but the time after 25 days in hyperthermophilic composting treatment was regarded as storage stage in this study. In both treatments, the main composting compartment or pile was diagonally split into 5 independent replicate piles (N=5). Based on the experience of the compost factory, five thermometers were placed in 40-50 cm depth for daily monitoring of the maximum fermentation temperatures.

The sample collection and DNA extraction

We collected samples from both composting treatments at days 0, 2, 4, 7, 9, 15, 21, 27, 33, and 45 as follows. To obtain well-distributed and homogenized samples, five subsamples per replicate segment were collected in 40-50 cm depth, mixed together (5000 g) and divided into two aliquots of which one was stored in liquid nitrogen for biological analyses and the other stored at 4 °C for physicochemical analyses. This sampling approach was chosen to reduce the potential bias caused by heterogeneity of the original composting substrate. The total genomic DNA was isolated using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) by following manufacturer's protocol. The DNA extraction was conducted three times for each sample and the DNA extracts were combined before the sequencing. The DNA content and the quality was checked with NanoDrop ND-2000 (Thermo Fisher Scientific, Wilmington, USA) and on 1% agarose gel.

Determination of physicochemical soil properties during composting

Following physicochemical properties were measured during the composting process

using methods described previously[32, 33]: pH, electrical conductivity (EC), water content (WC), total nitrogen content (TN), total carbon content (TC), total organic carbon content (TOC), total sulfur content (TS), inorganic carbon content (IC), electrical conductivity (EC) and ammonium (NH_4^+), and nitrate (NO_3^-) concentrations. Samples were oven-dried at 105 °C for 24 h to determine moisture content. EC and pH were determined using a conductivity meter (Radiometer, model CDM210) and a pH meter (PB-10, Sartorius, Germany), respectively. NH_4^+ and NO_3^- were measured by a continuous-flow autoanalyser (FlowSys, Systea, Rome, Italy). TOC and IC were quantified using an automatic TOC analyzer for liquid samples (Shimadzu TOC-L CPH, Kyoto, Japan). The TN, TC, and TS were determined with Elementar instrument (Vario MAX cube, Hanau, Germany) using dry combustion and the TN and TC values were used to calculate the C/N ratio.

Real-time quantitative PCR (qPCR) for determining antibiotic resistance gene and mobile genetic element abundances

Because tetracycline, macrolide, sulfonamide, and aminoglycoside resistance genes are the most abundant ARGs in the sewage sludge[23], we specifically chose to focus on these genes in this study (including ten tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetG*, *tetL*, *tetM*, *tetQ*, *tetO*, *tetW*, and *tetX*), six macrolide resistance genes (*ermB*, *ermF*, *ermT*, *ermX*, *mefA*, and *ereA*), seven aminoglycoside resistance genes (*aacA4*, *aadA*, *aadB*, *aadE*, *aphA1*, *strA*, and *strB*) and three sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*). We also measured changes in the abundance of five genes linked with mobile genetic elements such as integrases (*intI1*, *intI2*), plasmids (*ISCR1*, *IncQ*) and transposons (*Tn916/1545*, abbreviated as *Tn916*) and determined changes in bacterial cell densities by amplifying 16S rRNA gene copies using SYBR-Green real-time qPCR. The primers, annealing temperatures, and amplification protocols for all gene targets are listed in the supplementary material (Table S1). The qPCR and plasmid constructions were conducted according to a previous protocol[34] using the LightCycler 96 System (Roche, Mannheim, Germany). Briefly, the plasmids carrying target genes were obtained from TA clones and extracted using a TIAN pure Mini

Plasmid kit (Tiangen, Beijing, China). The standard plasmid concentrations (ng/mL) were determined with the Nanodrop ND-2000 (Thermo Fisher Scientific, Wilmington, USA) to calculate gene copy concentrations (copies/mL). The qPCR was carried out in 96-well plates containing 10 μL of GoTaq qPCR Master Mix (Promega, Madison, USA), 1.5 μL each of forward and reverse primers (4 mmol/L), 1 μL of template genomic DNA and 6 μL of nuclease-free water. Each qPCR run began with 2 min of initial denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 or 45 s according to the length of target at the primer-specific annealing temperature, and extension for 30 s at 72 °C. The amplification efficiencies of different PCR reactions ranged from 90% to 110% with R^2 values higher than 0.99 for all standard curves. Each reaction was run in triplicate along with standard curves and a negative control where the template genomic DNA was replaced with DNA-free water. The relative abundances of target genes are presented as gene copy numbers per 16S rRNA gene, while the absolute abundances of target genes are shown as gene copy numbers per gram dry sample.

High-throughput sequencing and bioinformatics analyses

To determine changes in bacterial community composition during composting, we amplified the V4-V5 region of the bacterial 16S rRNA gene using 515F/907R primers. The reverse primer contained a unique barcode for each sample and the DNA was amplified in triplicate before sequencing with Illumina Hiseq 2500 platform (Guangdong Magigene Biotechnology Co.Ltd, Guangzhou, China). Trimmomatic software (version 0.33) was used to trim the reads with low base quality. The high quality sequences were processed with QIIME pipeline to determine alpha and beta diversity[35]. The sequences were clustered into OTUs at 97% level similarity using Uclust clustering[36]. A set of representative sequences from each OTU were assigned taxonomically using a Ribosome Database Project Classifier with a confidence threshold of 0.80 as described previously[37]. Rarefaction curves were calculated to compare bacterial OTU diversity between different samples. The alpha diversity of each sample was determined as Chao1, Shannon, Observed species and Simpson

diversity indexes. Rarefaction curves were calculated to compare bacterial OTU diversity between different samples. The beta diversities of each composting treatment were analyzed with principal coordinate analysis (PCoA) based on Bray-Curtis distance matrix.

Correlation between different bacterial taxa and ARGs/MGEs during composting

We used local similarity analysis (LSA) to determine correlations between relative abundance of OTUs or annotated taxa and ARGs/MGEs during composting[38-40]. The LSA is an optimized method to detect non-linear, non-random, and time-sensitive relationships based on correlation networks[39, 40]. To reduce computing time and network complexity, only OTUs and taxa with relative abundance of 0.05% or higher were included in the analysis. Similarly, only highly significant ($P < 0.01$) cases with high local similarity scores were retained for further analysis. Finally, q-value (false-discovery rate, Benjamin Hochberg, $q < 0.01$) was applied to correct the *P*-values and to control the false-discovery rate for multiple comparisons. The retained LS interactions between ARGs and bacterial taxa were visualized as a network in Cytoscape v3.4.0 and network statistics analyzed with Network Analyzer as undirected networks using default settings[41].

Statistical analysis

A first-order kinetic model (ExpDec1) was used to fit the reduction in the abundance of target genes (gene copies per gram of dry sludge) during composting (Origin 9.0, Microsoft, USA)[19]. To analyze correlations between ARGs and bacterial taxa, PCoA (Bray-Curtis distance based), redundancy analysis (RDA), Adonis test, and Procrustes tests were performed in R 3.3.2 with vegan package v2.4-3. Effect Size (LEfSe) Linear Discriminant Analysis (LDA) was used to compare differences between conventional and hyperthermophilic composting at the genus level[42]. Discriminating features were identified using the following parameters: (1) the alpha value of factorial Kruskal-Wallis test between classes was set to 0.01 and (2) the threshold of the logarithmic LDA score was set to 2.0. Partial least squares path modeling

(PLS-PM) was employed to explore the direct, indirect and interactive effects between all measured variables for ARG abundances (The R package plspm (v 0.4.7))[43]. PLS-PM is a powerful statistical method to study interactive relationships among observed and latent variables[43, 44] and is widely applied to explain and predict relationships in multivariate data sets[44-46]. The model included the following variables: composting temperature, physicochemical composting properties (WC, TC, EC, pH, IC, C/N, TN, TOC, NH_4^+, NO_3^-), bacterial community composition (based on OTU abundances) and MGE and ARG abundances (relative target gene abundances, i.e., standardized by total bacterial abundances). Indirect effects are defined as multiplied path coefficients between predictor and response variables including all possible paths excluding the direct effect. The final model was chosen of all constructed models based on the Goodness of Fit (GoF) statistic - a measure of the model's overall predictive power.

Results

Hyperthermophilic composting is more efficient at removing ARGs and MGEs compared to conventional composting

The temperature profiles of the two composting treatments were clearly different (Figure S1). The temperature of hyperthermophilic treatment rapidly increased to about 90 ℃ after 24 hours of fermentation, while in the conventional composting, the temperature raised with much slower rate and reached maximum temperatures of 60 ℃ after 18 days of fermentation. All targeted 25 ARGs and 3 MGEs were detected in all samples; either of the plasmids (*ISCR1* and *IncQ*) was not detected in any of the samples. Mean concentrations of ARGs and MGEs were approximately 5.1×10^{11} and 1.1×10^{10} gene copies per gram (dry weight) of initial raw sludge, respectively, with tetracycline and sulfonamide resistance genes being the most dominant ARGs accounting for 64.8%-93.5% of all ARGs (Figure S2). At day 4, hyperthermophilic composting was more efficient at reducing aminoglycoside and macrolide resistance (64% and 84%, respectively) compared to conventional composting (31% and 41%, respectively, $P < 0.01$, Figure 1a). After 21 days of composting, the removal rates of

total ARGs and MGEs in hyperthermophilic composting (91 % and 88 %) were much higher compared to conventional composting (39 % and 51 %, $P < 0.05$, Figure 1b). During the 'storage phase' of hyperthermophilic composting (from day 27 to 45), abundances of ARGs increased in both treatments, but remained lower in hyperthermophilic compared to conventional composting ($P < 0.05$, Figure 1c). During the same period, MGEs remained at low abundances only in the hyperthermophilic composting, while increase in MGEs was observed in conventional composting ($P < 0.05$, Figure 1d). The residual amounts of ARGs and MGEs (relative abundances) were significantly lower in hyperthermophilic (0.05 and 0.002 copies/16S rRNA gene, respectively) compared to conventional composting (0.14 and 0.02 copies/16S rRNA gene, respectively, $P < 0.05$, Figure 1d). To compare the rate of ARG and MGE removal, we calculated target gene's half-life time ($t_{1/2}$) using a first-order kinetic model. We found that hyperthermophilic composting clearly shortened $t_{1/2}$ of all target resistance genes compared to conventional composting (Table 1). For example, the mean $t_{1/2}$ for ARGs and MGEs genes were 1.3 and 0.8 days in hyperthermophilic composting and 4.0 and 1.9 days in conventional composting, respectively.

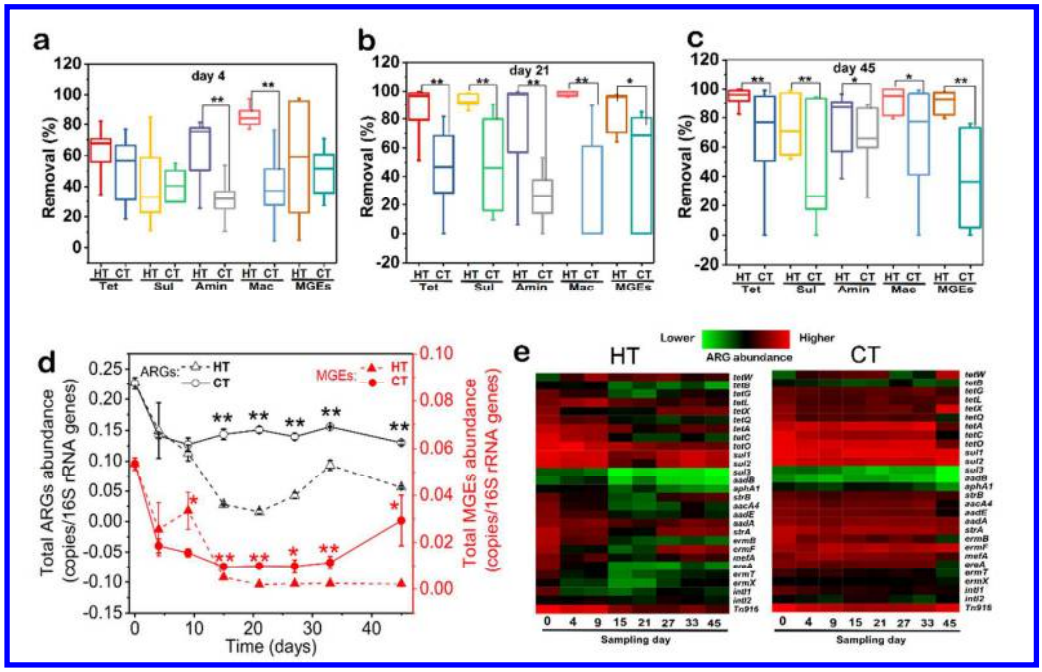


Figure 1. The removal of ARGs and MGEs during hyperthermophilic (HT) and conventional composting (CT). Panel (a-c): Boxplot figures showing the proportion and rate of removed ARGs and MGEs relative to day 0 in two composting treatments. Abbreviations on

X-axis indicate genes conferring resistance to tetracylines (Tet), sulfonamides (Sul), aminoglycosides (Amin), macrolides (Mac), and genes encoding mobile genetic element (MGEs). An asterisk (*) and two asterisks (**) indicate significant differences at 0.05 and 0.01 significance levels, respectively. Panel (d): The abundance dynamics of total ARGs (left Y-axis) and MGEs (right y-axis) in two composting treatments. Panel (e): Heat maps showing the mean abundance of normalized ARGs and MGEs (copies per 16S rRNA gene) in both composting treatments. Red and green colors indicate high and low gene abundances, respectively. All target gene abundances are shown as the relative abundances.

Hyperthermophilic and conventional composting leads to distinct bacterial communities

The two composting treatments selected for distinct bacterial communities during the 45 days of the experiment (Adonis test, $P < 0.001$), while no difference was observed at the last time point (at day 45; non-metric multidimensional scaling plot (NMDS): Figure 2a and PCoA analysis: Figure S3,). We also found that the bacterial community composition (at phylum level) varied more intensively in time under hyperthermophilic composting during the thermophilic phase (day 2 to 15, Figure 2c), while both total bacterial abundances (16S rRNA gene copy numbers) and bacterial community diversity were lower in hyperthermophilic compared to conventional composting especially ($P < 0.01$, Figure 2b, Figure S4). More specifically, hyperthermophilic composting reduced the relative abundance of Proteobacteria and Bacteroidetes from 32.1% to 2.0% and 30.6% to 0.32% by day 15, respectively (Figure 2c). Correspondingly, the abundance of thermophilic phyla, Thermi and Firmicutes (consisting principally of the class Bacilli), increased from 0.41% to 53.1% and from 8.0% to 42.3% by day 15, respectively (Figure 2c). As a result, the abundances of the two most dominant genera, *Thermus* (53.1%) and *Planifilum* (26.7%), belonging to Thermi and Firmicutes, were 86 and 37 times higher in hyperthermophilic compared to conventional composting (Figure 2d). The most dominant genera in the conventional composting were *Tepidimicrobium*, *Brachymonas*, *Actinomadura*, and *Acinetobacter*. These bacterial community structure differences were further confirmed by the linear discriminant analysis (LDA) effect size tool LEfSe (Figure 2d). Notably, Proteobacteria, including classes of

Gammaproteobacteria, Betaproteobacteria, and Alphaproteobacteria, were dominant discriminating key groups in the conventional treatment, whereas Thermi and Firmicutes, mainly including class Bacilli, were the key discriminating groups in the hyperthermophilic treatment (Figure S5). Towards the end of the experiment, the composition of bacterial communities became more similar (Figure 2c).

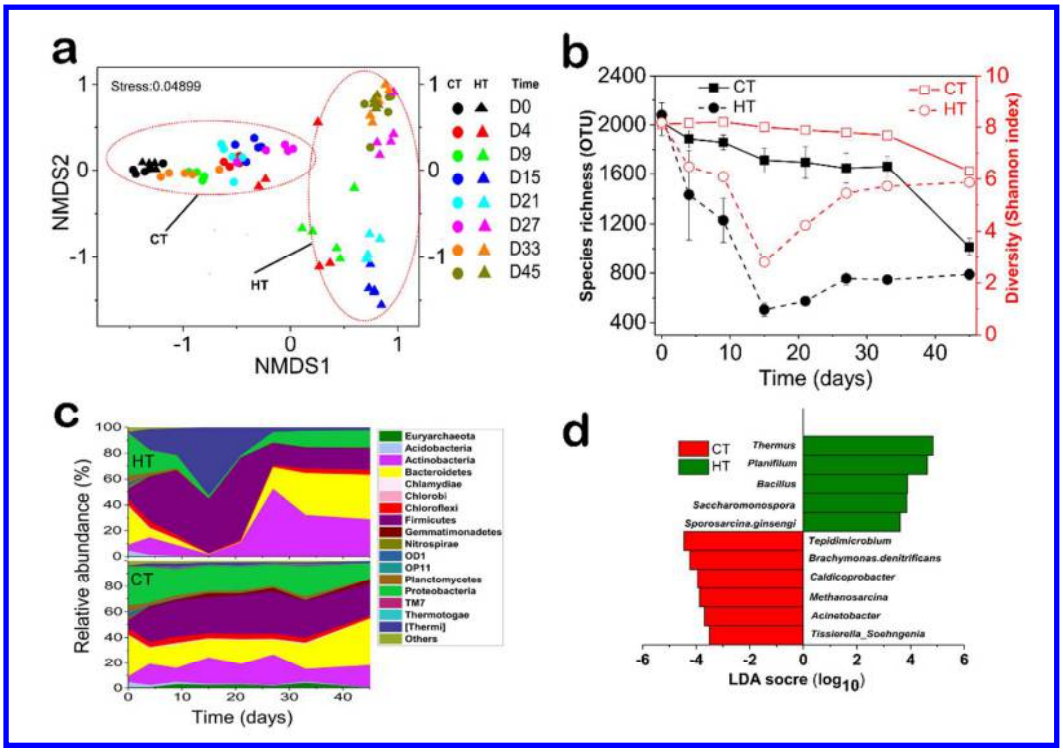


Figure 2. Changes in bacterial community composition and diversity under hyperthermophilic (HT) and conventional composting (CT). Panel (a): The overall distribution pattern of OTU-based bacterial community dissimilarity in the two composting treatments (based on non-metric multidimensional scaling (NMDS); ordination derived from weighted-UniFrac distances). Circles denote for conventional and triangles for hyperthermophilic composting and different colors denote for different sampling days. Panel (b): Changes in bacterial community species richness (left Y-axis) and alpha diversity (Shannon index; right Y-axis) in the two composting treatments. Panel (c): The relative abundance of different bacterial phyla in the two composting treatments. (d): Histogram of the LDA scores for discriminating bacterial genera that showed clear abundance differences between hyperthermophilic and conventional composting treatments (genus level, LDA-score > 3.5).

Correlations between ARG, MGE and bacterial taxa abundances

Based on procrustes analysis, gene abundances of ARGs were significantly correlated with the bacterial community composition in both composting treatments (Figure S6).

Similarly, ARGs and MGEs were significantly correlated with each other ($P < 0.001$) in both composting treatments (Figure S7). Local similarity and network analysis to link ARGs, MGEs and bacterial taxa abundances revealed that most ARGs and MGEs correlated significantly ($P < 0.01$) with 52 and 31 bacterial taxa (at genus level) within conventional (Table S2) and hyperthermophilic (Table S3) composting treatments, respectively. Of all ARG-associated bacteria, 17 genera were common for both treatments, 14 genera were only detected in the hyperthermophilic and 35 were detected only in the conventional composting treatment (Figure 3a). More than 50% of bacteria that significantly correlated with ARGs and MGEs belonged to Proteobacteria and Bacteroidetes, the two dominant taxa in initial raw sludge samples (Figure S8). Interestingly, the densities of *Acinetobacter*, *Dokdonella*, and *Fusibacter* correlated with both ARG and MGE abundances in both composting treatments, while *Methanobacterium* (archaea) densities correlated with ARGs and MGEs only in the hyperthermophilic composting. ARGs and MGEs were significantly clustered in the networks ($P < 0.01$, Figure S9). For example, the cluster of resistance genes around *intI1* and *intI2*, and *Tn916* ($P < 0.01$) consisted of known gene cassettes associated with MGEs. Together these results suggest that bacterial taxa that correlated positively with ARGs and MGEs could have played an important role for the proliferation of resistance genes during composting.

We next focused on comparing the associations between ARGs, MGEs and bacterial taxa in both composting treatments. The majority of ARG-associated bacteria (17.9% of all sequences) in the initial raw sludge belonged to *Acinetobacter* (2.3%), *Bacteroides* (4.0%), *Dechloromonas* (4.5%), *Nitrospira* (3.1%), and *Paludibacter* (3.8%, Table S4-5). The abundance of these taxa decreased more in the hyperthermophilic compared to the conventional treatment during the composting (Figure 3b). A similar trend was also found at the family level: the mean abundance of ARG-associated bacteria belonging to families Moraxellaceae, Bacteroidaceae, Rhodocyclaceae, Nitrospiraceae, and Porphyromonadaceae sharply decreased from 46.6% to 15.3% in the hyperthermophilic treatment after 4 days of composting (Figure 3b). The densities of these bacteria remained low (<5%) throughout the

experiment (from day 4 to 45) in the hyperthermophilic composting, while the relative abundances of those taxa were maintained at an elevated level (30%-48.3%) until day 33 in the conventional composting treatment ($P < 0.05$, Figure 3b). Together these results suggest that hyperthermophilic composting reduced the abundance of potential ARG bacterial host taxa more efficiently compared to conventional composting.

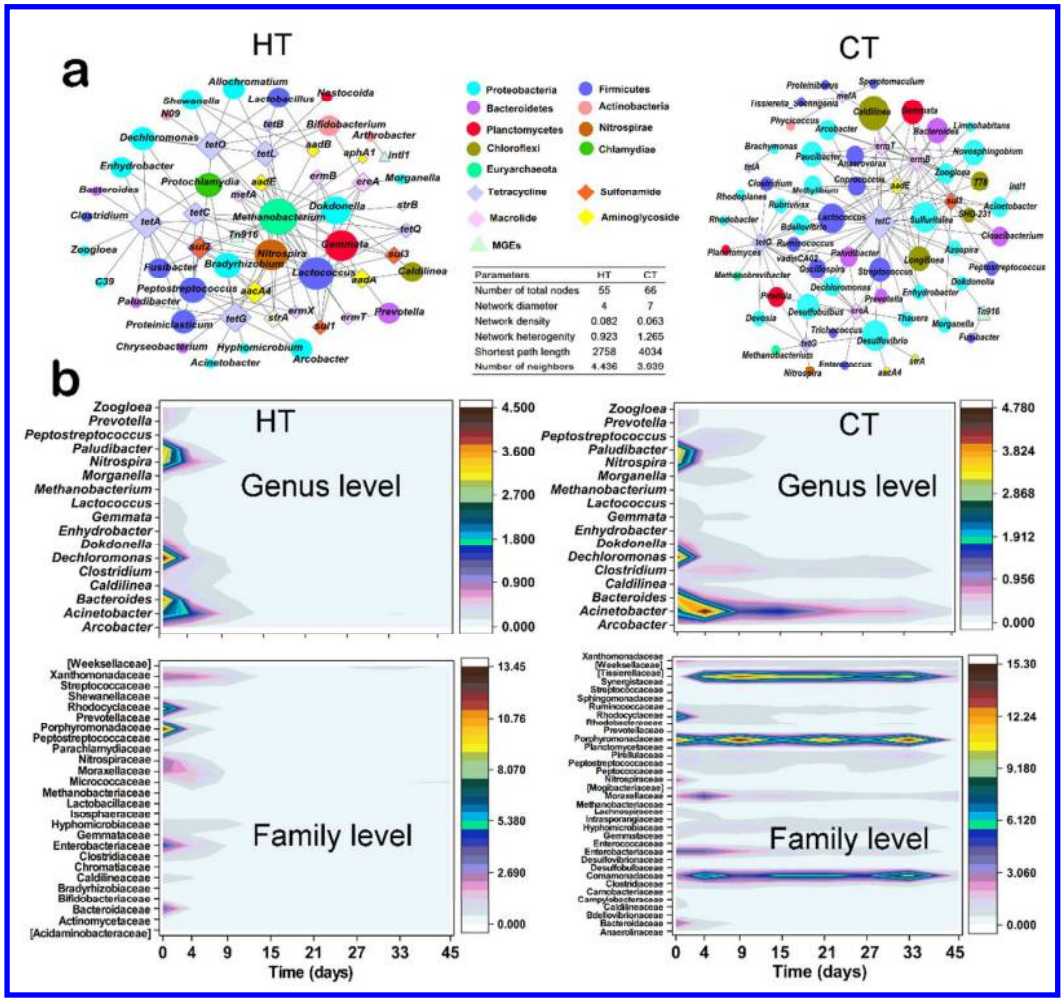


Figure 3. The relationship and the abundance of ARG- and MGE-associated bacteria during hyperthermophilic (HT) and conventional (CT) composting. Panel (a): Co-occurrence network analysis showing the associations between ARGs/MGEs and bacterial taxa in both composting treatments. Panel (b): Distribution profiles showing the relative abundance of ARG- and MGE-associated bacteria at genus (upper panels) and family (lower panels) level in both composting treatments. The legend on the left side denote for taxonomic groups and the legend on the right side the relative bacterial abundances (%) based on total 16S rRNA gene sequences for each presented taxa. The network analysis of all gene abundances are based on the relative abundances.

444

445 **Determining the direct and indirect relationships between composting**
446 **temperature, physicochemical composting properties, bacterial community**
447 **composition and MGE abundance for the abundance of ARGs**

448 The RDA analysis explained 89.7% and 73.0% of the total variance of ARG
449 abundances in hyperthermophilic and conventional composting treatments,
450 respectively (included variables: composting temperature and properties, bacterial
451 community composition and MGE abundances, Figure S10). To explore the effects of
452 composting temperature, composting properties, bacterial community composition
453 and MGEs on the ARG abundances in more detail, we constructed a partial least
454 squares path model (PLS-PM) to assess the direct and indirect effects between
455 observed (indicators) and latent constructs (Figure 4). We found that composting
456 temperature had similar positive or negative direct effects on composting properties,
457 bacterial community composition and ARG and MGE abundances in both composting
458 treatments (Figure 4). However, the link between temperature and MGE abundances
459 was only significant in the hyperthermophilic composting. Composting properties had
460 only significant negative direct effects on the bacterial community composition in
461 both treatments, while the bacterial community composition had significant positive
462 direct effects on the abundances of MGEs and ARGs in both treatments. Crucially,
463 MGE abundances strongly explained the ARG abundances in the hyperthermophilic
464 composting, while the direct effect of bacterial community composition was more
465 important factor in the conventional composting (Figure 4a-b). These results suggest
466 that ARG abundances were affected by different mechanisms in hyperthermophilic
467 and conventional composting treatments.

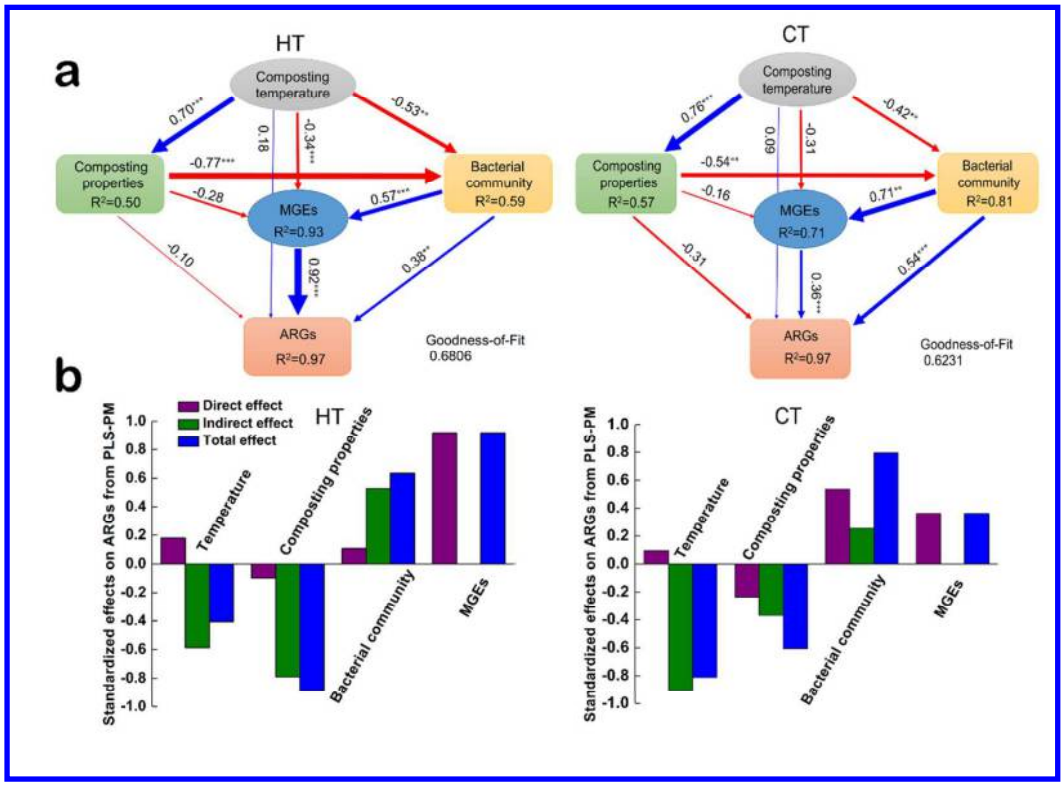


Figure 4. Partial least squares path model (PLS-PM) showing the direct and indirect effects of different factors on ARG abundances in hyperthermophilic (HT) and conventional composting (CT). Panel (a): PLS-PM describing the relationships between temperature, composting properties, bacterial community composition and MGE abundances on ARG abundances in hyperthermophilic and conventional composting. Larger path coefficients are shown as wider arrows and blue and red colors indicate positive and negative effects, respectively. Path coefficients and coefficients of determination (R^2) were calculated after 999 bootstraps and significance levels are indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). The Goodness of Fit (GoF) for the hyperthermophilic and conventional treatments was 0.68 and 0.62, respectively. Panel (b): Standardized direct and indirect mean effects derived from the partial least squares path models. All target gene abundances are presented as relative abundances.

Discussion

Hyperthermophilic composting is more effective at reducing ARG and MGE abundances compared to conventional composting

Temperature played a crucial role for the rate and level of ARG and MGE removal in our experiment. It is well known that high temperature is the principal factor controlling the inactivation of pathogenic microorganisms in organic waste[47]. Despite large temperature differences, no significant difference in total ARG abundances were

488 observed before day 4 between the two composting treatments. This suggests that
489 degradation of ARGs needs a longer exposure at high temperatures. Relatively long
490 incubation period at over 70 °C temperature in thermophilic composting treatment (15
491 days) might thus have been important factor contributing the high ARG-removal rate.
492 Although the maximum temperature of traditional composting reached up to 60 °C
493 (>55 °C for approximately 5 days), most of the quantified ARGs still persisted and
494 some ARGs even increased in abundance in time (Figure 1c). This persistence of
495 ARGs could be due to the presence of some heat tolerant hosts of ARGs or horizontal
496 transfer of ARGs via MGEs. Laboratory studies have suggested that temperatures
497 above 70 °C are required to completely and directly degrade bacterial DNA[48], which
498 could explain our observed increased removal of ARGs and MGEs in
499 hyperthermophilic compared with conventional composting. In addition, antibiotic
500 residues in the waste and composting products could have affected the emergence of
501 ARGs[49, 50]. However, most antibiotics degrade very rapidly ($t_{2/1}$=0.9 to 9 days) in
502 thermophilic composting according to previous studies[51, 52]. Crucially, we used the
503 same raw materials for conventional and hyperthermophilic composting, and hence,
504 the effect of potential antibiotic residues unlikely affected the difference in
505 ARG-removal in this study. We also found that the $t_{1/2}$ of all tested target genes was
506 shortened in hyperthermophilic compared to conventional composting, and in the case
507 of genes *intI1*, *Tn916*, *tetB*, and *sul1*, the $t_{1/2}$ of most ARGs and MGEs was lower than
508 previously reported[19, 53, 54]. Together these results suggest that hyperthermophilic
509 composting was more efficient at removing ARGs and MGEs.
510 While the abundances of ARGs remained lower in hyperthermophilic compared to
511 conventional composting, the abundances of ARGs also increased during the 'storage
512 stage' of the hyperthermophilic composting (Figure 1d). This could have been caused
513 by regrowth of certain bacterial ARG hosts due to a decrease in the composting
514 temperature (Figure 1d). However, this increase in ARG abundances was not
515 associated with an enrichment of MGEs (Figure 1d), which suggests that this
516 secondary ARGs dissemination was not driven by horizontal gene transfer. From a
517 practical perspective, this result suggests that composting products should not be

stored for extended periods of time, in our case of weeks, due to risk of increase in ARGs abundances. In particular, some ARGs such as *tetX*, *tetW*, *sul1*, *sul2*, and *ermF* were still found in reasonably high abundances in the compost, suggesting that they are extremely tolerant to high temperatures, or alternatively, can use thermophilic bacteria as their hosts. This is in line with previous studies showing that some heat tolerant ARGs are not removed during composting[19, 23], and hence, some complementary strategies are needed to attain complete removal of all types of ARGs. Among five tested MGEs, genes encoding two integrases (*intI1* and *intI2*) and one transposon (*Tn916*) but not any plasmid genes (*ISCR1*, *IncQ*) were detected in any of the samples. This suggests that horizontal gene transfer of ARGs was mainly driven by integrases and transposons. In the future, higher numbers of MGEs and ARGs should be studied using high-throughput quantitative PCR approaches to build a more complete picture of the role of horizontal gene transfer for the resistome during composting. Our findings suggest that the temperature applied in conventional composting was likely not high enough to degrade ARGs and MGEs directly. Instead, the reduction of ARGs and MGEs was probably caused by decrease in the abundance of ARG and MGE hosting bacteria[55, 56]. In contrast, periodically extremely high temperatures could have directly broken down ARGs and MGEs during hyperthermophilic composting. This idea is also supported by the PLS-PM results that revealed direct effects of hyperthermophilic composting on ARGs and MGEs (Figure 4).

Hyperthermophilic composting alters the bacterial community composition and ARG-bacterial taxa associations

NMDS analysis revealed that the bacterial community composition differed between conventional and hyperthermophilic composting until day 33, but no difference was observed at day 45 (the end). This suggest that bacterial communities converged between two composting treatments when the composting treatments reached similar temperatures and physicochemical properties[57]. Compared to conventional composting, hyperthermophilic composting led to reduced total bacterial abundances and lowered species richness and bacterial community diversity (Figure 2b and Figure

S4). These effects could have important indirect effects on ARGs and MGEs. First, the reduction in total bacterial densities could have constrained the horizontal transfer of ARGs via less frequent encounter rates[58]. Second, loss of diversity could have resulted in the reduction of suitable ARG and MGE host bacteria. In line with these hypotheses, we found that bacteria belonging to two phyla (Figure 3), Proteobacteria and Bacteroidetes that are common hosts of ARGs, were dominant in the raw sludge[59, 60], but observed at significantly reduced abundances in the hyperthermophilic treatment (Figure S8b). According to previous studies[59, 61], the majority of the bacteria (>50%) associated with ARGs and MGEs belonged to Proteobacteria and Bacteroidetes. In contrast, extreme thermophiles belonging to the genera *Thermus* and *Planifilum* dominated (89% relative abundance, Figure 2c) the thermophilic phase of the hyperthermophilic composting. Crucially, both genera are not associated with ARGs or MGEs[62]. Even though hyperthermophilic and conventional composting resulted in a distinct bacterial community composition (Figure 2c), this difference gradually decreased towards the later stages of the composting when the temperature of both treatments fell back to normal. Crucially, even though the abundance of Proteobacteria and Bacteroidetes increased during the later stages of hyperthermophilic composting, the abundance of ARGs increased only slightly, while an obvious increase in ARGs abundances was observed in the conventional composting (Figure 1e). One reason for this is that most of the potentially ARGs-linked bacterial host taxa were killed during the extremely high-temperature composting phase. Alternatively, reduction in the diversity and abundance of horizontal gene transfer agents (MGEs) could have constrained further reinfection of suitable hosts. To study the associations between ARGs and bacterial taxa in more detail, we performed combined bacterial network and LSA analysis, which are powerful tools to indirectly explore potential co-dependencies based on co-occurrence relationships[40]. In agreement with previous studies[63-65], we found that *Bacteroides*, *Clostridium*, *Enterococcus*, and the archaeon *Methanobrevibacter* were positively associated with ARGs. These potential ARG hosts were strongly reduced in the hyperthermophilic treatment, suggesting that these potential ARG hosts were killed

578 during the composting (Figure 3a). This conclusion was further confirmed using the
579 relative abundance data obtained from high-throughput sequencing for each host
580 (Figure 3b). Conversely, the dominant genera in conventional composting were
581 *Brachymonas*, *Acinetobacter*, *Tissierella_Soehngenia* that all were positively
582 associated with ARGs or MGEs. Together these results suggest that both density- and
583 diversity-mediated effects improved the removal of ARGs in hyperthermophilic
584 composting by reducing the occurrence of horizontal gene transfer and by directly
585 killing potential ARG-host bacteria.
586
587 **Hyperthermophilic and conventional composting had potentially different**
588 **underlying mechanisms for ARG-removal**
589 To explore complex relationships between composting temperature, composting
590 properties, bacterial community composition and MGE abundances on ARG
591 abundances, we conducted a PLS-PM analysis. We found that ARG abundances were
592 not directly affected by composting temperature. This was contradicting our
593 hypothesis that composting temperature was the main and direct contributor of ARGs
594 reduction. However, it is in line with a previous study showing that the bacterial
595 community rather than the composting temperature was the major direct factor
596 affecting the abundance of ARGs[23]. Our model suggests that underlying mechanisms
597 behind the ARG-removal were different for hyperthermophilic and conventional
598 composting. More specifically, MGE abundances had strongest direct influence on
599 ARG abundances in hyperthermophilic composting. In contrast, bacterial community
600 composition was the major determinant of ARG abundances in the conventional
601 composting. However, in both treatments, bacterial community composition and
602 MGE abundances were significantly correlated with composting temperature (Figure
603 4), and most importantly, showed correlations in the same direction even though the
604 magnitude was different. This suggests that both MGEs and the bacterial community
605 composition determined the ARG abundances in both composting treatments but that
606 the relative importance of these factors was different. In hyperthermophilic
607 composting, ARG abundances appeared to be more strongly limited by less frequent

horizontal gene transfer as MGEs were almost completely removed. In contrast, the dynamics and the abundance of potential bacterial hosts played a more important role in conventional composting. Based on our PLS-PM analyses (Figure 4a), MGEs were shown to by direct transfer agents of ARGs and no indirect effects were found. However, other factors including composting temperature, composting properties, and bacterial community composition had a profound effect on ARGs which were partly direct (e.g. in hyperthermophilic composting) or indirect via changes in the bacterial community composition (conventional composting). Most ARG cassettes are found in MGEs such as integrons located on transposons and broad-host range plasmids[66]. We also found that most bacterial taxa were associated with more than one ARG subtype (Figure 3b) and that ARGs and MGEs were highly correlated in both treatments (Figure S7). This further supports the idea that ARGs were carried on MGEs that could have mobilized ARGs between different bacterial taxa.

In conclusion, this study demonstrates that hyperthermophilic composting is an efficient and powerful methodology for decreasing ARGs and MGEs compared to conventional composting. Mechanistically, this was likely driven by direct negative effects of the high temperature on the stability of ARGs and MGEs and direct or indirect negative effects on bacterial abundances and relative abundance of potential ARG-host bacteria. Our results also suggest that the relative importance of MGEs was more important in hyperthermophilic composting, while the role of the bacterial community composition was more important for conventional composting on ARG-removal. Hyperthermophilic composting thus represents a promising biotechnology for reducing the abundance of ARGs before solid waste land application.

Supporting Information

The temperature profile of two composting treatments; absolute abundances of ARG and MGE; principal coordinate analysis; bacterial density and alpha diversity; taxonomic cladogram; procrustes analysis; correlation between absolute ARG and MGE abundances; abundance of potential ARG hosts; network analysis for patterns among ARGs and MGEs; redundancy analysis;

information of PCR primers; additional details on local similarity analysis.

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Notes

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814 **Table 1 First-order kinetic model analysis showing the half-lives ($t_{1/2}$) and kinetic coefficients**
815 **(k) for different ARGs and MGEs in hyperthermophilic (HT) and conventional (CT)**
816 **composting.**

Target	HT			CT		
ARG/**MGE**	$t_{1/2}$ (day)	k	R^2	$t_{1/2}$ (day)	k	R^2
tetA	2.43	0.33	0.84	/	/	/
tetB	1.20	0.70	0.89	2.98	0.39	0.57
tetC	1.27	0.55	0.99	1.58	0.52	0.99
tetG	1.59	0.49	0.88	2.85	0.54	0.93
tetL	0.098	2.58	0.50	0.12	10.95	0.69
tetQ	/	/	/	2.10	0.60	0.97
tetO	/	/	/	0.77	1.12	0.99
tetX	/	/	/	1.08	0.90	0.83
sul1	1.60	0.62	0.60	2.38	0.72	0.83
sul2	1.17	0.80	0.58	1.72	0.62	0.96
sul3	2.49	0.32	0.83	8.46	0.10	0.86
strA	1.64	0.53	0.73	2.46	0.49	0.94
strB	1.49	0.60	0.51	2.64	0.55	0.60
aacA4	1.35	0.54	0.96	3.80	0.41	0.88
aadA	1.60	0.61	0.67	2.59	0.62	0.91
aadB	2.15	0.47	0.70	1.90	0.58	0.91
aadE	1.18	0.62	0.98	3.15	0.43	0.97
aphA1	1.66	0.53	0.84	2.25	0.77	0.66
ermB	0.80	0.87	0.99	1.16	0.98	0.69
ermT	0.62	1.17	0.97	2.38	0.45	0.91
ermX	0.96	0.93	0.55	8.74	0.49	0.68
mefA	0.91	0.90	0.80	31.54	0.00	0.73
ereA	1.15	0.60	0.98	0.67	2.61	0.75

intI1	**0.55**	**1.40**	**0.91**	**2.37**	**0.37**	**0.97**
Tn916	**1.01**	**0.76**	**0.89**	**1.70**	**0.48**	**0.99**

Note: For a better fitting model, first order kinetic mode ($t_{2/1}$) is based on data using absolute abundances of target genes from day 0 to 33.